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Simultaneous detection of native and invasive crayfish and *Aphanomyces astaci* from environmental DNA samples in a wide range of habitats in Central Europe

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Abstract

Crayfish of North American origin are amongst the most prominent high-impact invasive invertebrates in European freshwaters. They contribute to the decline of European native crayfish species by spreading the pathogen causing crayfish plague, the oomycete *Aphanomyces astaci*. In this study we validated the specificity of four quantitative PCR (qPCR) assays, either published or newly developed, usable for environmental DNA (eDNA) screening for widely distributed native and non-native crayfish present in Central Europe: *Astacus astacus*, *Pacifastacus leniusculus*, *Faxonius limosus* and *Procambarus virginalis*. We then conducted an eDNA monitoring survey of these crayfish as well as the crayfish plague pathogen in a wide variety of habitat types representative for Central and Western Europe. The specificity of qPCR assays was validated against an extensive collection of crayfish DNA isolates, containing most crayfish species documented from European waters. The three assays developed in this study were sufficiently species-specific, but the published assay for *F. limosus* displayed a weak cross-reaction with multiple other crayfish species of the family Cambaridae. In the field study, we infrequently detected eDNA of *A. astaci* together with the three non-native crayfish species under examination. We never detected eDNA from *A. astaci* together with native crayfish, but in a few locations eDNA from both native and non-native crayfish was captured, due either to passive transport of eDNA from upstream populations or co-existence

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in the absence of infected crayfish carriers of *A. astaci*. In the study, we evaluated a robust, easy-to-use and low-cost version of the eDNA sampling equipment, based mostly on items readily available in garden stores and hobby markets, for filtering relatively large (~5 l) water samples. It performed just as well as the far more expensive equipment industrially designed for eDNA water sampling, thus opening the possibility of collecting suitable eDNA samples to a wide range of stakeholders. Overall, our study confirms that eDNA-based screening for crayfish and their associated pathogen is a feasible alternative to traditional monitoring.

Keywords
crayfish plague, eDNA monitoring, eDNA sampling methods, quantitative PCR, TaqMan assay validation

Introduction

Environmental DNA (hereafter eDNA) is commonly defined as genetic material obtained directly from environmental samples (soil, sediment, water) without any obvious signs of the biological source material (Thomsen and Willerslev 2015). In water samples, eDNA typically originates from single-celled uncultured microorganisms or, in the case of multicellular taxa, from shed cells, faeces, mucus, body fluids, gametes, spores or other propagules (Strand et al. 2014; Deiner et al. 2016; Mächler et al. 2016) or even from recently dead and decomposing organisms (Strand et al. 2019).

During the past decade, different concepts of eDNA analyses have become established for various purposes such as monitoring endangered and elusive targets, invasive species, as well as parasites and pathogens (Kirshtein et al. 2007; Thomsen et al. 2012a; Takahara et al. 2013; Rusch et al. 2018; Strand et al. 2019). There are two essentially different approaches to eDNA monitoring: either broad spectrum metabarcoding for bio-assessments of whole communities (Thomsen et al. 2012a; Valentini et al. 2016; Ruppert et al. 2019) or more targeted approaches for the detection and quantification of one or several species of interest (Jerde et al. 2011; Thomsen et al. 2012b), usually using species-specific quantitative real-time PCR (qPCR) or droplet digital PCR (ddPCR). Since eDNA has a relatively short half-life in the water column of aquatic systems (Dejean et al. 2011), positive detection suggests that the targeted organism is either present or has been present within the system very recently.

One of the pathogens for which monitoring methods based on eDNA have been developed is the oomycete *Aphanomyces astaci* Schikora, the causative agent of crayfish plague (Strand et al. 2011, 2012, 2014; Robinson et al. 2018; Wittwer et al. 2018). Since its initial introduction into Europe in the late 1850s (Alderman 1996), and reinforced by subsequent introductions of several Non-Indigenous Crayfish Species (NICS) of North American origin (Holdich et al. 2009), crayfish plague has ravaged the continent and led to mass mortalities of native crayfish (Alderman 1996; Holdich et al. 2009). *Aphanomyces astaci* is usually carried as a benign infection by its natural crayfish hosts from North America, where both originate. However, crayfish indig-

enous to Europe usually lack efficient defence mechanisms to resist this pathogen and thus whole populations tend to be eliminated as a result of crayfish plague outbreaks (Söderhäll and Cerenius 1999; Holdich et al. 2009; Vrålstad et al. 2014). This explains why *A. astaci* is a disease listed by the World Organisation for Animal Health (OIE 2019) and featured on the list of the "world's 100 worst invasive species" (Lowe et al. 2004).

American crayfish species, such as the spiny cheek crayfish *Faxonius limosus* (Rafinesque, 1817), the signal crayfish *Pacifastacus leniusculus* (Dana, 1852) and the red swamp crayfish *Procambarus clarkii* (Girard, 1852), were originally introduced into Europe for stocking or aquaculture purposes (Holdich et al. 2009). Others, such as the marbled crayfish *Procambarus virginalis* Lyko, 2017, reached European waters through the pet trade (Chucholl 2013; Kouba et al. 2014). All species listed above pose a threat to native European crayfish species and are therefore subject to the EU Regulation on the prevention and management of the introduction and spread of invasive alien species (Regulation (EU) No 1143/2014).

The marbled crayfish, *P. virginalis*, is causing great concern outside of Europe, too. This triploid species seems to have emerged as a thelytokous parthenogenetic form of *Procambarus fallax* (Hagen, 1870), possibly from the pet trade (Gutekunst et al. 2018; Martin et al. 2010). Thus, it produces female-only offspring and a single individual is required to establish a new population. It has been shown to thrive in a very broad range of habitats, recently demonstrated in Madagascar (Andriantsoa et al. 2019).

When non-indigenous crayfish are present, the only conceivable option to eradicate crayfish plague is by treating the entire waterbody with pesticides such as Betamax-VET (Sandodden and Johnsen 2010). This procedure kills the crayfish hosts and subsequently also the crayfish plague pathogen which depends on its host for long-term survival (Söderhäll and Cerenius 1999). However, this is only applicable to smaller aquatic habitats (Peay et al. 2019) and, even there, it is an extremely costly and devastating undertaking, often not compliant with local legislation. Therefore, mitigation strategies must be employed to preserve and protect Indigenous Crayfish Species (ICS) and their natural environment. These mitigation strategies can include the prohibition of fishing in certain areas or the enforcement of decontamination protocols for fishing gear. They could also encompass the creation and management of the so-called "ark sites", where introduction of neither the alien crayfish nor the disease is likely (Peay 2009a). When creating such ark sites or planning restocking and rescue transfers, precise knowledge about the distribution of crayfish plague vectors and presence or absence of the crayfish plague agent in the vicinity is required. For this purpose, the eDNA methodology is a particularly suitable tool (Cowart et al. 2018; Strand et al. 2019).

Recent research has focused on developing eDNA monitoring for early alert of NICS and *A. astaci*, as well as for efficient biomonitoring of ICS. The main goals are safeguarding indigenous crayfish while limiting the spread of both NICS and crayfish plague pathogen (Strand et al. 2014, 2019; Agersnap et al. 2017; Cai et al. 2017; Vrålstad et al. 2017; Harper et al. 2018; Wittwer et al. 2019).

In this study we demonstrate the applicability of eDNA-based screening for crayfish and the crayfish plague pathogen in a wide range of aquatic habitats in Czechia, a Central European country with a long tradition of crayfish conservation and research. Three European crayfish species, the noble crayfish *Astacus astacus* (Linnaeus, 1758), the stone crayfish *Austropotamobius torrentium* (Schrank, 1803) and the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823) are found in local waters. The two former species are native to the country, the latter being introduced from Eastern Europe to multiple localities in the late 19th century (Štambergová et al. 2009). Crayfish plague has caused large-scale mortalities of native crayfish in the area since the 1890s (Kozubíková et al. 2006). Although not considered a conservation problem throughout most of the 20th century, crayfish plague outbreaks, caused by *A. astaci* genotypes associated with different North American host taxa (Grandjean et al. 2014), are at present rampant in the country (Kozubíková et al. 2008; Kozubíková-Balcarová et al. 2014; Mojžišová et al. 2020).

Czech waters host three documented North American crayfish species. *Faxonius limosus* that invaded the Elbe river as far back as the 1960s (Petrusek et al. 2006) and *P. leniusculus*, introduced for fishery purposes in 1980 (Filipová et al. 2006), are both widespread in at least some regions of the country (Kouba et al. 2014; Mojžišová et al. 2020). *Procambarus virginalis* has recently been documented from two sites, most likely resulting from aquarium releases (Patoka et al. 2016), but there is a high probability that other established populations of *P. virginalis* are yet waiting to be discovered. All these species are confirmed carriers of *A. astaci* (Svoboda et al. 2017). Infections of Czech populations have been documented for *P. leniusculus* and *F. limosus* (Kozubíková et al. 2009), but not for *P. virginalis* (Patoka et al. 2016).

Native and non-native crayfish populations can be found in a wide range of diverse habitats in Czechia: large and smaller rivers and streams as well as artificial still waters including fishponds, flooded quarries and reservoir lakes. There is a wealth of documented data on existing crayfish populations in lentic and lotic waterbodies in the country (Štambergová et al. 2009; Svobodová et al. 2012), together with data on the infection status by *A. astaci* in NICS populations (Kozubíková et al. 2009, 2011). Thus, Czechia is a suitable region to conduct a study focusing on eDNA-based detection of multiple NICS and their pathogen across a broad range of habitats.

The goal of the study presented here is two-fold: firstly, to validate the specificity of presumably species-specific qPCR assays for selected native and non-native crayfish present in Central Europe (Fig. 1). Three assays newly developed for this study and one previously published assay were tested against a broad panel of DNA isolates from various crayfish species present in Europe or available via the ornamental pet trade. Secondly, the presence of the crayfish plague agent *A. astaci* as well as its various crayfish hosts by means of eDNA analysis of water samples was evaluated. These were collected from various Czech localities and some from urban waters from Berlin (Germany) and Budapest (Hungary), which are representative for crayfish habitats in Central and Eastern Europe.

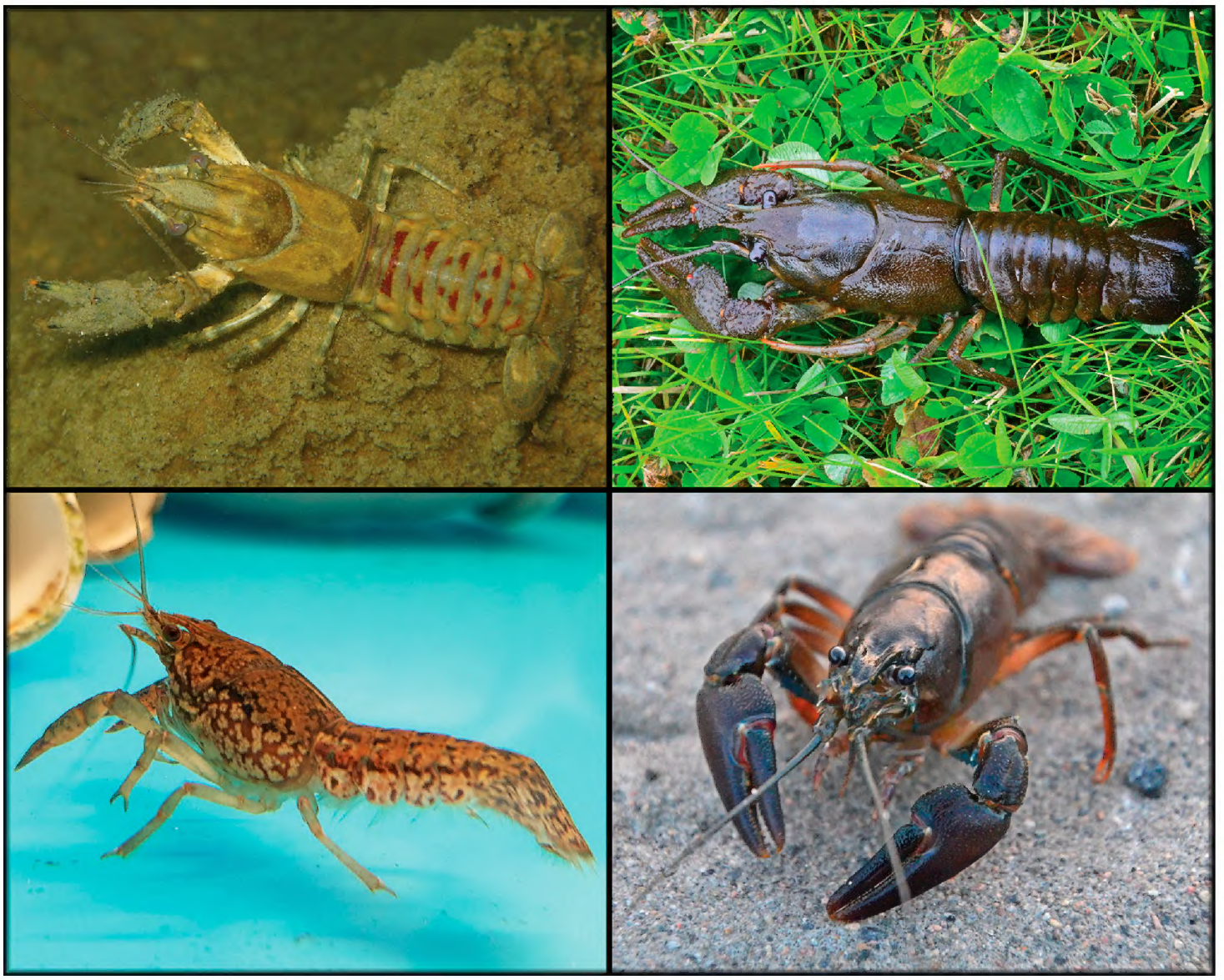

Figure 1. Crayfish species searched for by means of eDNA. Species clockwise from top left: *Faxonius limosus*, *Astacus astacus*, *Pacifastacus leniusculus*, *Procambarus virginalis*. Photos taken by A. Petrusek (Fl) and J. Rusch (Aa, Pl, Pv).

Methods

Study sites and populations

A full range of all relevant habitats for Central and Western Europe was covered, including large rivers and small streams, a thermal stream, natural lakes and man-made reservoirs, flooded quarries and fishponds (in total 32 localities; Suppl. material 1: Table S1). The majority of the samples (28) were taken in August 2017 at various waterbodies within Czechia, for which previous presence of crayfish was reliably known or presumed. The sampling sites were chosen to ensure that each one could be considered negative for at least some of the four target crayfish species, i.e. *F. limosus*, *P. virginalis*, *P. leniusculus* and *A. astacus*. None of the sites was within the known distribution area for stone crayfish in the country (Vlach et al. 2009; Petrusek et al. 2017a). Two samples were collected in December 2018 at two lakes in Berlin with a recently reported or assumed presence of both *P. virginalis* and *F. limosus* (Linzmaier et al. 2018; A. Mrugała, pers. comm.). Two additional water samples were obtained in January 2019 from a

stream in Budapest and its thermal tributary with a confirmed co-existence of the same two (and also additional) NICS (Szendőfi et al. 2018; A. Kouba, pers. comm.). Control eDNA samples were collected from an aquarium housing numerous marbled crayfish individuals, held at the Czech University of Life Sciences, Prague.

For comparison with eDNA results, crayfish were actively searched for at most sampling locations by manual examination of suitable shelters to confirm their *in-situ* presence. At the Czech sites containing NICS, we also attempted to obtain individuals to test for infection with *A. astaci*. After collection of samples for eDNA analysis, these crayfish were either captured directly at the sampling site on the same date or obtained from a nearby site within the same watercourse. Occasionally, we benefited from availability of such samples from previous recent fieldwork, assuming that the infection status of the NICS population does not change dramatically in a short time (Matasová et al. 2011). Crayfish plague diagnostics were carried out according to the method described in Vrålstad et al. (2009) with minor modifications (Mrugała et al. 2015). In brief: the soft abdominal cuticle and part of the tail fan of each crayfish were dissected and ground in liquid nitrogen. Total genomic DNA was then extracted using the DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf, Germany) and the DNA extracts were then screened using the qPCR protocol for detection of *A. astaci* described below.

eDNA sample collection and extraction

Water samples at Czech locations 1 to 28 were obtained according to Strand et al. (2019) by filtering up to 5 l of water through glass fibre filters (47 mm AP25 Millipore, 2 µm pore size; Millipore, Billerica, USA), utilising a portable peristaltic pump (Masterflex E/S portable sampler; Masterflex, Cole-Parmer, Vermon Hills, USA), tygon tubing (Masterflex) and an in-line filter holder (Millipore). The front end of the tube was fastened to the inside of a plastic box which was weighted with lead on the bottom. This box was lowered into the water between 2 m and 5 m from the water's edge or to the centre of smaller streams. Before the filter was placed into the holder, water was pumped through the tubes for several minutes to remove any sediments that could have been disturbed from the waterbed and thus prevent clogging of the filter (Strand et al. 2019). For sampling sites where less than 5 l of water was filtered due to filter clogging, the final volume is noted in Table 2. At each location, two filter samples were taken.

For the samples obtained at locations 29 to 32 (Berlin and Budapest) the same filters (47 mm AP25 Millipore, 2 µm pore size) were used. However, the filters were placed into filter cups (Nalgene Analytical Test Filter Funnel, 145-0045; Thermo Fisher Scientific, Waltham, USA) after removal of the original filter provided by the manufacturer. Pumping was carried out by attaching the provided filter-cup adapter to a ¾ inch garden water hose and a drill-operated pump (product code 1490-20; Gardena, Ulm, Germany) (Fig. 2). As opposed to the protocol described above, the filters and filter cups were submerged into the water since they were situated at the front end of the pumping system (Fig. 2). The samples from the aquarium with *P. virginalis* and from the Barát stream in Budapest (sites 31, 32) were obtained after transporting water

Figure 2. Drill-powered sampling equipment. The low-cost sampling equipment used in this study consisting of a drill-powered pump, single use forceps, filter cups and glass fibre filters. The pump depicted in the bottom right corner is one of many alternative models to the one used in this study.

from the location in disinfected 5 l containers. This water was stored in the dark at low temperatures but not frozen and was filtered upon arrival in the laboratory using the drill-operated pumping system described above.

Filters from locations 1 to 28 were submerged in 4 ml of cetyl trimethyl ammonium bromide (CTAB) buffer in individual 15 ml Falcon tubes immediately after filtration and subsequently stored on ice until their arrival at the laboratory where they were stored at –20 °C prior to further analysis. Filters from locations 29 to 32 were placed into separate zip-lock bags containing ca. 70 g of silica gel following Carim et al. (2016), which ensured efficient desiccation, and stored in an opaque container until further analysis in the laboratory.

To prevent contamination of filters and accidental spreading of crayfish plague, a strict disinfection protocol was followed at each location. After filtering, all the equipment was submerged in, and filled with, a 10% chlorine bleach solution for a minimum of 15 minutes to break down any vital pathogen spores and residual eDNA. Then the tubes and filter holders were rinsed with a 5% sodium thiosulphate ($Na_2S_2O_3$) solution to neutralise the chlorine solution. Prior to water sample filtration, the equipment was thoroughly rinsed with ambient water from the sampling site. While using the drill-operated pumping system, separate tubing and filter holders were used at each respective sampling site, thus eliminating the concern for carryover contamination.

DNA isolation from the filters was performed according to the CTAB method described in Strand et al. (2019). In brief: the samples were lysed on CTAB buffer and proteinase K at 65 °C for one hour, cleaned and separated with chloroform and then precipitated in isopropanol. The pellets were then re-suspended in TE-buffer.

Due to the large volume of eluate from each filter, the samples were split up into two subsamples (technical replicates) to bypass the volume restrictions caused by centrifuge size. These subsamples were subsequently processed separately. Each extraction process incorporated an environmental blank control and an extraction blank control as a precautionary measure to detect any potential contamination during the extraction (Strand et al. 2019).

Molecular detection of target species with qPCR

Molecular eDNA detection of all five target-species (the crayfish plague pathogen *A. astaci* and the crayfish *A. astacus*, *P. leniusculus*, *F. limosus* and *P. virginalis*) was based on TaqMan MGB qPCR assays, either published in the case of *A. astaci* (Vrålstad et al. 2009) and *F. limosus* (Mauvisseau et al. 2018) or developed in this study (*A. astacus*, *P. leniusculus* and *P. virginalis*).

Due to the absence of any published assay for *P. virginalis* while this study was being carried out, we designed a qPCR assay with species-specific primers and a minor grove binder (MGB) probe targeting the mitochondrial gene for the cytochrome c oxidase subunit I (COI) of this asexually reproducing, genetically uniform species (cf. GenBank reference sequence: JF438007). We have since learnt of the existence of a newly-published assay (Mauvisseau et al. 2019) which targets a very similar fragment of the COI gene and thus differs only marginally from the one developed by us.

High specificity of the primers–probe combination was first ensured by checking the variation of the potential primer and probe sites against COI sequences of all crayfish known to occur in European waters, both native and invasive, and various related crayfish species of the family Cambaridae, particularly those available from the pet trade (taxa listed in Suppl. material 2: Table S2). This was accomplished using Geneious version 11.0.1 (Biomatters Limited, Auckland, New Zealand) and MEGA 7.0.26 (Kumar et al. 2016) through visual comparison. The efficacy of the primers and probe was evaluated using the Primer Express software (Version 3.0.1, Applied Biosystems, Foster City, USA).

New assays, differing from those published in Agersnap et al. (2017), Dunn et al. (2017), Larson et al. (2017), Harper et al. (2018), Mauvisseau et al. (2018) and Robinson et al. (2018), were designed for *A. astacus* and *P. leniusculus*. These two assays were developed with particular regard to functionality on both the qPCR and the droplet digital PCR (ddPCR) platform (D.A. Strand, unpublished). However, in this study we have only tested the efficiency and efficacy of the assays on the qPCR platform. Sequences from individual crayfish from several European regions (including North American individuals for *P. leniusculus*; Petrusek et al. 2017b) obtained from GenBank were used to design the assays for *A. astacus* and *P. leniusculus*.

For *in-vitro* validation, to determine the specificity of the assays, we re-used a total of 29 DNA isolates from tissues of crayfish species from previous studies on diversity of both indigenous and non-indigenous crayfish species in Europe that involved COI

Table 1. Primers and probes used in the present study. The probes used are TaqMan MGB probes with either FAM or VIC reporter dyes.

Target species	Target marker	Primer/probe	Sequence (5'-3')	Reference
Aphanomyces astaci	ITS	forward	AAGGCTTGTGCTGGGATGTT	Vrålstad et al. (2009)
		reverse	CTTCTTGCGAAACCTTCTGCTA	Vrålstad et al. (2009)
		probe	FAM-TTCGGGACGACCC-MGBNFQ	Vrålstad et al. (2009)
Astacus astacus	COI	forward	CCCCTTTRGCATCAGCTATTG	current study
		reverse	CGAAGATACACCTGCCAAGTGT	current study
		probe	FAM-CTCATGCAGGCGCAT-MGBFNQ	current study
Pacifastacus leniusculus	COI	forward	GAGTGGGTACTGGATGAACTG	current study
		reverse	GAAGAAACACCCGCTAAATGAAG	current study
		probe	VIC-CAGCGGCTATTGCT-MGBFNQ	current study
Faxonius limosus	COI	forward	CCTCCTCTCGCTTCTGCAAT	Mauvisseau et al. (2018)
		reverse	AACCCCTGCTAAATGCAACG	Mauvisseau et al. (2018)
		probe	FAM-CTCATGCAGGGGCATCAGTGG-MGBFNQ	Mauvisseau et al. (2018)
Procambarus virginalis	COI	forward	ACGGGCAGCTGGTATAACTATG	current study
		reverse	TCTCCTCCACCAGCAGGATC	current study
		probe	FAM-CCGCTATTTGTTTGGTCAGTA-MGBNFQ	current study

sequencing (Filipová et al. 2011; Chucholl et al. 2015; Petrusek et al. 2017a). We also used isolates from surveys of *A. astaci* infections in various carrier species (Tilmans et al. 2014; Mrugała et al. 2015) and crayfish plague outbreaks (Kozubíková-Balcarová et al. 2014) (see additional material, Suppl. material 2: Table S2). The identity of non-indigenous species was confirmed and variation at the target marker (COI) in most of these particular isolates was assessed by DNA barcoding in previous studies (Filipová et al. 2011; Mrugała et al. 2015). The isolate collection, used to test the assay specificity, contained most of the native crayfish known from Western, Central and Northern European countries and the Balkans (see distribution maps in Kouba et al. 2014), with the exception of narrowly-endemic lineages related to *A. torrentium* (Klobučar et al. 2013; Pârvulescu 2019) and the thick-clawed crayfish *Pontastacus pachypus* (Rathke, 1837).

Both newly-developed assays for *A. astacus* and *P. leniusculus*, as well as the published assay for *F. limosus* (Mauvisseau et al. 2018), were subjected to the same *in-vitro* validation procedure as the assay for *P. virginalis*, described above. To ensure optimal performance of all qPCR assays targeting crayfish, we determined the most suitable annealing temperatures through a temperature gradient from 56 °C to 63 °C and multiple primer-probe concentrations were evaluated. Our two objectives were to define the conditions when the assays show efficient amplification of the target DNA but minimal cross-reaction with DNA of related taxa and, if possible, to establish a common protocol for routine application of all assays.

The final protocol used for eDNA screening was identical for the detection of all four crayfish species. The undiluted and diluted samples were run in the following 25 µl reaction: 12.5 µl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, USA), 1.25 µl of each 10 µM primer (forward and reverse), 1.25 µl of 5 µM TaqMan MGB probe, 3.75 µl of PCR-grade water and 5 µl of DNA sample. The following qPCR cycling conditions were used: an initial denaturation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 1 min.

For all species-specific crayfish assays, we followed recommendations for defining the limit of detection (LOD) and the limit of quantification (LOQ) in qPCR assays used for diagnostic analyses of genetically-modified organisms and microbiological pathogens in foodstuff, tissues and environmental samples (Berdal et al. 2008). These have also been used for previously-published assays for crayfish plague (Vrålstad et al. 2009) and freshwater crayfish ("the Norwegian approach" in Agersnap et al. 2017). Genomic DNA from all target species was extracted according to the protocol in Agersnap et al. (2017) and stock solutions of 50 $ng.\mu l^{-1}$ genomic DNA (measured using Qubit fluorometer; Invitrogen, Carlsbad, USA) from each species were used to prepare a four-fold dilution series of 13 standard dilutions. In an initial qPCR test, ≥ 3 replicates of the standard dilution 1–8 were run on a Stratagene Mx3005P with qPCR-conditions as described above, while the standard dilutions 9–13 were run in 20 replicates. A template concentration of approximately 1 DNA copy per PCR volume will yield a positive:negative ratio of 7:3 (70% detection success; Berdal et al. 2008). Thus, the copy number in the standard dilutions closest to 70% detection rate were then calculated with most probable number (MPN) calculations (Berdal et al. 2008) and the obtained copy number was then used to calculate copy numbers in the more concentrated standards. The LOD was established for each assay following the criteria that LOD is the lowest concentration that yields a probability of false negatives < 5% (Berdal et al. 2008; Vrålstad et al. 2009). The LOQ was established using the same acceptance level as set for qPCR quantification of the crayfish plague pathogen *A. astaci* (Vrålstad et al. 2009), with observed standard deviation < 0.5 for the Ct-values.

In order to detect *A. astaci* in both eDNA samples and crayfish tissues, we used the assay developed by Vrålstad et al. (2009) with modifications according to Strand (2013). Each undiluted and diluted sample was run in the following 25 µl reaction: 12.5 µl of TaqMan Environmental Master Mix 2.0, 2.5 µl of each 5 µM primer (forward and reverse), 1 µl of 5 µM TaqMan MGB probe, 1.5 µl of PCR-grade water and 5 µl of DNA sample. The following qPCR cycling conditions were used: an initial denaturation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 15 s and annealing at 62 °C for 30 s.

All qPCR analyses of the eDNA samples were carried out on an Mx3005P qPCR thermocycler (Stratagene, San Diego, USA) at the Norwegian Veterinary Institute, Oslo. The validation of crayfish assays concerning specificity tests against other crayfish species was performed on a BioRad iQ5 (Bio-Rad, Hercules, USA) thermocycler at the Faculty of Science, Charles University, Prague. An analysis of a subset of eDNA

isolates on the BioRad iQ5 thermocycler suggested comparable performance to that on Mx3005P.

As described above, each filter was divided into two technical replicates/subsamples. Both subsamples were analysed as 2x undiluted and 2x 10-fold diluted replicates, in total 4 qPCR replicates per filter. Results for each respective filter were considered positive, only if more than one of the four reactions yielded positive results. A cut-off value was set at Ct 41 following previous recommendations (Agersnap et al. 2017; Kozubíková et al. 2011; Strand et al. 2019) which means that any amplification occurring at or above this value was not considered a positive detection.

The presence or absence of qPCR inhibition was controlled by calculating the difference in Ct values (ΔCt) between the undiluted and corresponding 10-fold diluted DNA replicates as described in Kozubíková et al. (2011) and Agersnap et al. (2017). In case of apparent inhibition (if $\Delta Ct < 2.82$) the estimated eDNA copy number was based on the 10-fold diluted DNA replicates alone, while if $\Delta Ct > 3.82$ (i.e. 10-fold dilution out of range), the estimation of eDNA copy number was based solely on the undiluted DNA replicates (see Suppl. material 4: Table S4 for observed inhibition). If none or only one of the relevant replicates were detected above LOQ, further quantification was not performed and thus qPCR inhibition was not possible to evaluate either.

Results

Optimising and validating the crayfish qPCR assays

We successfully developed new assays for *A. astacus*, *P. leniusculus* and *P. virginalis*. All three assays were apparently species-specific *in-silico* and, for the first two, we also confirmed this *in-vitro*. The assay for *P. virginalis* displayed weak cross-amplification of three other cambarid species (see below). While *in-silico* testing the assays and comparing sequences of the respective crayfish to their closest relatives, we observed the assay for *F. limosus* to differ from a closely-related species *Faxonius* cf. *virilis* (a lineage of the *F. virilis* complex known from Europe; Filipová et al. 2010) by only one mismatch in the forward primer and two mismatches in the probe and the reverse primer, respectively. For subsequent qPCR testing with a temperature gradient, we included DNA isolated from European *F.* cf. *virilis* (labelled *F. virilis* below). While using the PCR conditions (annealing temperature 56 °C) suggested by the authors (Mauvisseau et al. 2018), *F. limosus* and *F. virilis* DNA were amplified at Ct 17.92 and 24.62 respectively. An increase in annealing temperature to 60.5 °C resulted in amplification of *F. limosus* and *F. virilis* DNA at Ct 18.58 and 34.12 respectively, thus increasing the specificity of the assay, although still cross-reacting with *F. virilis*.

Ensuing specificity testing against the collection of all DNA isolates (Suppl. material 2: Table S2) was carried out at 60 °C. The assay for *F. limosus*, which amplified the DNA of the target taxon at Ct 17.7 to 18.5, also amplified DNA of isolates of the following species (lowest Ct stated): *F. virilis* (Ct 30.14), *F. margorectus* (Ct 36.32),

F. rusticus (36.74), *F. harrisonii* (Ct 40.72), *F. punctimanus* (Ct 40.86), *P. virginalis* (Ct 36.13), *P. zonangulus* (Ct 37.91) and *P. acutus* (Ct 35.79). The assay for *P. virginalis*, which amplified the DNA of the target taxon at Ct 18.3 to 23.33 (depending on the starting DNA concentration of isolate), also weakly cross-amplified DNA of isolates from *P. acutus* (Ct 37.29), *P. alleni* (Ct 38.22) and *P. clarkii* (Ct 39.41).

For all crayfish assays, LOD was experimentally established as 5 copies/PCR reaction with good margin; the observed detection success for 20 replicates of a standard dilution corresponding to ~2–4 copies per PCR reaction was between 90–100% (for details see Suppl. material 3: Table S3). Further, LOQ was established as 10 copies per PCR reaction, where the assays demonstrated acceptable repeatability with observed standard deviation for the Ct-values (Suppl. material 3: Table S3).

Environmental DNA monitoring

We detected eDNA of all surveyed crayfish species during our sampling effort (Fig. 3). We also detected eDNA of the crayfish plague pathogen *A. astaci* together with the three investigated non-native crayfish species, but only infrequently. More commonly, eDNA from non-native crayfish was detected alone (Fig. 3, Table 2). A full overview of the qPCR results and eDNA copy estimations is supplied in Suppl. material 4: Table S4.

From the total of 32 surveyed locations, eDNA from native *A. astacus* was unambiguously detected in seven (~22 %) locations. In two of these, however, a positive amplification only occurred in one out of two filter samples. At four locations, the eDNA results were corroborated by observation of *A. astacus* at the sampling sites (Table 2). Simultaneous detection of *A. astacus* and *F. limosus* eDNA was observed in two locations (7 – Všechlapy reservoir and 10 – Pšovka), eDNA from *A. astacus* and *P. leniusculus* was simultaneously detected in location 16 (Oslava). Environmental DNA from the crayfish plague pathogen *A. astaci* was never detected in samples that contained *A. astacus* eDNA. However, in location 10 (Pšovka), we caught specimens of *F. limosus*, whose tissue analyses showed low *A. astaci* prevalence (20%) and very low infection load (agent level 2, A2; Vrålstad et al. 2009).

Non-native *P. leniusculus* was detected by eDNA in eight locations (25%), all where the species was expected according to our prior knowledge (Suppl. material 1: Table S1). All detections occurred in both samples taken at the respective sampling sites. The eDNA results were corroborated by observation of signal crayfish at seven locations on the date of sampling. Environmental DNA from the crayfish plague pathogen *A. astaci* was detected in only two of the locations where *P. leniusculus* was detected (13 – Malše and 15 – Dračice). In these two locations, data from tissue analyses confirmed high prevalence (80% and 100%) and low to high infection load (up to A3 and A5, respectively). For three other *P. leniusculus* positive locations (16 – Oslava, 20 – Žďárka, and 22 – Staviště), the apparent absence of *A. astaci* eDNA was corroborated by no detection of the pathogen in screened crayfish individuals (Table 2). Generally, *P. leniusculus* was the only crayfish species detected through eDNA at the respective

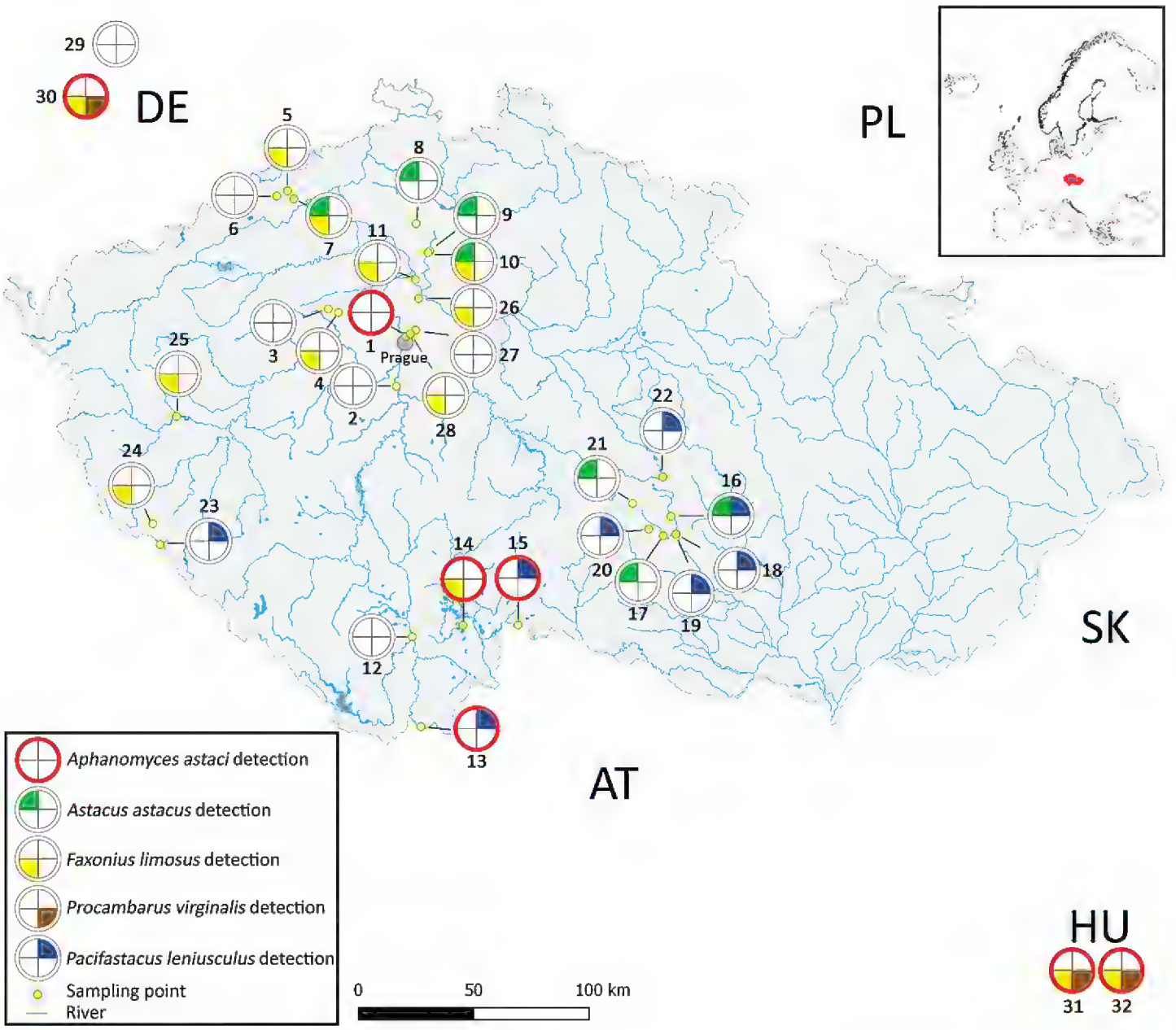

Figure 3. Map of Czechia with results of the eDNA screening at the sampling locations. Blue lines and areas represent the main water bodies, yellow dots represent each respective sampling point with numbers referring to the sampling sites in Table 2 and Suppl. material 1: Table S1. Pie charts: the red ring around the pie charts indicates unambiguous eDNA detection of *A. astaci* whereas a white ring represents non-detection. The green colour indicates detection of *A. astacus*, blue indicates detection of *P. leniusculus*, yellow represents detection of *F. limosus* and brown indicates presence of *P. virginalis*. The neighbouring countries are indicated by their two-letter ISO codes: AT, DE, HU, PL and SK stand for Austria, Germany, Hungary, Poland and Slovakia.

sampling points, except at location 16 (Oslava) where eDNA of *A. astacus* was also detected. Environmental DNA from *P. leniusculus* never co-occurred with other non-native crayfish species.

Environmental DNA of non-native *F. limosus* was unambiguously detected in 13 locations. At one location the detection occurred on only one filter. *In-situ* observation on the day of sampling confirmed the eDNA results at eight locations. Environmental DNA from the crayfish plague pathogen *A. astaci* was detected in four of the *F. limosus*-positive locations, three of which were urban waters of Berlin (site 30 – Hundekehlesee) and Budapest (31 and 32 – Barát); presence of infected crayfish was confirmed at site no.

Table 2. Results of the eDNA analyses from individual sampling sites. Volumes of water filtered (in l) indicated. The target species are abbreviated as follows: AA for *Astacus astacus* (noble crayfish), PL for *Pacifastacus leniusculus* (signal crayfish), PV for *Procambarus virginalis* (marbled crayfish), FL for *Faxonius limosus* (spiny-cheek crayfish) and Aph for *Aphanomyces astaci* (crayfish plague agent). The column labelled "obs" indicates any crayfish observed at the respective site during the sampling, using the same species abbreviations. Sites where manual search for crayfish was impossible to conduct are indicated by "ns". Detection in eDNA samples is stated as unambiguous confirmation on 0 (marked as "–"), 1 or 2 filters per site (for more details, see Suppl. material 4: Table S4). The prevalence of *A. astaci* in NICS populations and maximum agent level in infected crayfish following Vrålstad et al. (2009) is specified. For more details about the sampling sites and specific comments, including past evidence of crayfish presence, see Suppl. material 1: Table S1.

No.	Locations	Habitat	Volume (in l)	qPCR positives in eDNA samples						*A. astaci* screening in NICS	
				AA	PL	FL	PV	Aph	obs	Prevalence	Max. agent level
1	Vltava in Prague	River	4	–	–	–	–	2		88% (15/17)	A4
2	Vltava (Vrané)	Reservoir	2.2	–	–	–	–	–		n/a	
3	Kněžák Pond	Fishpond	1.35	–	–	–	–	–		n/a	
4	Smečno	Urban pond	1.9	–	–	1	–	–		n/a	
5	Barbora	Flooded mine	10	–	–	2	–	–	FL	0% (0/22)	(3 x A1)
6	Osecký Pond	Fishpond	0.7	–	–	–	–	–		n/a	
7	Bouřlivec (Všechlapy)	Reservoir	2.8	1	–	2	–	–	ns	n/a	
8	Liběchovka	Stream	1.5	2	–	–	–	–		n/a	
9	Pšovka (above Harasov)	Stream	4.4	2	–	–	–	–	AA	n/a	
10	Pšovka (Harasov)	Pond out	10	1	–	2	–	–	FL	20% (3/15)	A2
11	Elbe	River	3.8	–	–	2	–	–	FL	35% (6/17)	A4
12	Malše in České Budějovice	River	1.85	–	–	–	–	–		n/a	
13	Malše (border with Austria)	Stream	10	–	2	–	–	2	PL	80% (16/20)	A3
14	Zlatá stoka	Channel	1.6	–	–	2	–	1		12.5% (1/8)	A3
15	Dračice	Stream	1.2	–	2	–	–	2	PL	100% (20/20)	A5
16	Oslava (upstream)	Stream	2.3	2	2	–	–	–	PL	0% (0/23)	A0
17	Balinka (upstream)	Stream	4	2	–	–	–	–	PL	n/a	
18	Oslava (confluence)	Small river	10	–	2	–	–	–	PL	n/a	
19	Balinka (confluence)	Stream	4.1	–	2	–	–	–		n/a	
20	Žďárka	Stream	5.1	–	2	–	–	–	PL	0% (0/28)	A0
21	Ochozský Brook	Stream	0.85	2	–	–	–	–	AA	n/a	
22	Staviště	Stream	4.4	–	2	–	–	–	PL	0% (0/18)	A0
23	Kouba	Stream	3	–	2	–	–	–	PL	n/a	
24	Starý Klíčov – Lomeček	Quarry	10	–	–	2	–	–	ns	n/a	
25	Mže (Hracholusky)	Reservoir	3.2	–	–	2	–	–	FL	29% (2/10)	A3
26	Kojetice	Quarry	10	–	–	2	–	–	FL	70% (14/20)	A2

No.	Locations	Habitat	Volume (in l)	qPCR positives in eDNA samples						*A. astaci* screening in NICS	
				AA	PL	FL	PV	Aph	obs	Prevalence	Max. agent level
27	Prague–Prosek (park)	Urban pond	10	–	–	–	–	–		n/a	
28	Rokytka	Stream	2	–	–	2	–	–		n/a	
29	Krumme Lanke	Lake	10	–	–	–	–	–	ns	n/a	
30	Hundekehlesee	Lake	10	–	–	2	1	1	ns	n/a	
31	Tributary of Barát	Thermal stream	10	–	–	2	2	2	FL, PV	85% (17/20)	A3
32	Barát Brook	Stream	10	–	–	2	2	2	FL, PV	n/a	

31. In four locations (10 – Pšovka, 11 – Elbe, 25 – Mže and 26 – Kojetice), data from *F. limosus* tissue analyses confirmed *A. astaci* prevalence ranging from low to high (20%, 35%, 29% and 70% respectively) and very low to moderate infection load (A2, A4, A3 and A2), but no *A. astaci* spores were detected by eDNA there. Environmental DNA of *F. limosus* and native *A. astacus* was detected together in two locations (mentioned above; Table 2, Fig. 3). *Faxonius limosus* eDNA did not co-occur with that of other non-native crayfish species in Czechia, but did so at both locations in Budapest (31 and 32) and one location in Berlin (30 – Hundekehlesee) (Table 2, Fig. 3). These three urban waters were the only sites where we confirmed eDNA of *P. virginalis* (in all cases together with *A. astaci*). Specimens of this crayfish species were observed at the Hungarian sampling sites.

In 24 subsamples (i.e. technical replicates), eDNA of *A. astaci* was detected (with Ct values in the qPCR reaction not exceeding 41; Suppl. material 4: Table S4), but it was quantifiable only in 12 subsamples. Four of these detections (33%) showed inhibition, mostly weak. *Astacus astacus* eDNA was detected in 27 subsamples of which 17 were above the LOQ. Two of these (12%) displayed weak inhibition. All of the 32 subsamples that were positive for *P. leniusculus* were quantifiable and none of them showed any inhibition. Of 49 subsamples positive for *F. limosus*, ten were quantifiable (above LOQ) and four (40%) showed some inhibition. Ten subsamples were positive for *P. virginalis* of which eight were quantifiable; ΔCt values for these subsamples indicated some qPCR inhibition as well.

Discussion

Crayfish eDNA and assays – our study compared to the state of art

This study explores the use of the eDNA methodology for the detection of the crayfish plague pathogen *A. astaci* and freshwater crayfish in Central and Western Europe, simultaneously covering several species and numerous habitat types. A steadily increasing number of studies use eDNA monitoring to assess the presence of native crayfish or the introduction and spread of non-native crayfish across the globe (Tréguier et al. 2014; Dougherty et al. 2016; Ikeda et al. 2016, 2019; Agersnap et al. 2017; Larson

et al. 2017; Mauvisseau et al. 2018). In Europe, these tend to be complemented by screening for the accompanying conservationally relevant pathogen *A. astaci* (Robinson et al. 2018; Mauvisseau et al. 2019; Strand et al. 2019; Wittwer et al. 2019).

One of the potential pitfalls of eDNA monitoring methods, relying on species-specific qPCR, lies within the development and testing of the assays themselves. Specificity testing, both *in silico* and *in vitro* against isolates of any closely-related species that may cause false-positive results, is therefore imperative. While several previous studies have performed specificity testing on a limited range of locally relevant freshwater crayfish species (Dougherty et al. 2016; Agersnap et al. 2017) and one on a more comprehensive range of non-target species than just those found in the examined area (Larson et al. 2017), we tested the assays used for *A. astacus*, *P. leniusculus*, *P. virginalis* (this study) and *F. limosus* (Mauvisseau et al. 2018) towards most native and non-native freshwater crayfish species known from European waters (Suppl. material 2: Table S2). The three former assays proved sufficiently specific, although a weak cross-amplification with other cambarids was observed when testing the *P. virginalis* assay against DNA isolates from other crayfish. However, the *F. limosus* assay yielded a relatively strong non-target amplification for *F. virilis* with the originally recommended annealing temperature (56 °C). An increase of the annealing temperature to 60 °C reduced its extent, but DNA of several other *Faxonius* and *Procambarus* species also yielded cross-amplification with this assay. We may presume that at 56 °C this effect would be substantially stronger.

The cross-amplification of non-target species at high Ct levels, close to cut-off of both assays for *F. limosus* and *P. virginalis*, should pose no practical problems in eDNA studies, as these were observed while analysing tissue isolates. Environmental samples contain, by their very nature, less DNA of the target species than tissue isolates and thus usually amplify more than 10 cycles later compared to DNA isolates from tissue. A false-positive detection is therefore highly unlikely to occur for most of these taxa, possibly with the exception of *F. virilis* detection by the *F. limosus* assay. Yet, it seems that achieving universal specificity for assays may pose a challenge, especially in regions with higher crayfish species biodiversity than Europe where closely-related species can co-occur that differ only marginally in the target DNA marker. In such cases it may be beneficial to apply the metabarcoding approach with general primers to better capture the overall crayfish biodiversity (Thomsen et al. 2012a).

However, for management purposes in Europe, even the non-specific amplification of *F. virilis* is not likely to pose a substantial problem as non-native *F. virilis* has so far only been found in London (Ahern et al. 2008) and the Netherlands (Soes and van Eekelen 2006). Moreover, even in the case of such a false detection, this still indicates the presence of an invasive crayfish of concern to the EU (Regulation (EU) No 1143/2014) that may act as a crayfish plague carrier (Tilmans et al. 2014).

Environmental DNA monitoring of crayfish – pros and cons

An increasing number of studies, including the present one, demonstrate that the eDNA approach is effective in providing presence/absence data for freshwater crayfish

(Dougherty et al. 2016; Ikeda et al. 2016, 2019; Agersnap et al. 2017; Mauvisseau et al. 2018, 2019; Strand et al. 2019). In contrast to the crayfish plague agent *A. astaci*, where it is possible to determine the rough quantity of spores in the water (Strand et al. 2011, 2012, 2014; Makkonen et al. 2013; Svoboda et al. 2013, 2014), it is not possible to quantify crayfish biomass, population density or population structure on the basis of eDNA detection (Dougherty et al. 2016; Agersnap et al. 2017; Laurendz 2017; Rice et al. 2018).

For conservation purposes, for example when determining the suitability of an unpopulated habitat as an ark site, the critical information is nevertheless the presence or absence of the crayfish plague pathogen and any potential vectors thereof. For this purpose, eDNA monitoring provides an efficient alternative for confirming the presence of target organisms (Strand et al. 2019). However, caution must be exercised regarding the interpretation of samples that do not yield any positive detection. Many samples and large volumes should be analysed to substantiate the high likelihood of absence of a rare target organism convincingly (Strand et al. 2014, 2019).

In this study, we failed to detect *A. astaci* eDNA in four of eight locations where crayfish tissue analyses confirmed the presence of this pathogen, albeit in either a low prevalence or low infection load. Here, we have no knowledge about the density of the carrier-population, but the combination of low pathogen prevalence and low crayfish population density is obviously a challenge to reveal *A. astaci* presence in a random water sample. At location 29 (Krumme Lanke), we were unable to detect eDNA of any of the five target organisms despite reports of the presence of both *F. limosus* and *P. virginalis* somewhere in the lake in the recent past (Linzmaier et al. 2018). This might be explained by spatial mismatch (Harper et al. 2018) and low ambient temperatures which may have led to decreased activity of crayfish (Bubb et al. 2004; Rusch and Füreder 2015) and thus decreased emission of eDNA.

Dilution of the eDNA amount in large waterbodies is a factor that may lead to the failure to detect the target taxa, even if present. This is also exemplified in location 1 (the river Vltava in Prague) where we detected the crayfish plague agent but none of the host species. At this sampling site, the Vltava is more than 115 m wide and the flow rate on the date of sampling was ~50 m^3/s, so any eDNA signal would be subject to significant dilution, a common problem reported in previous studies (Strand et al. 2014, 2019). The presence of *F. limosus* in the Vltava in Prague has previously been confirmed, with crayfish displaying high levels of infection with *A. astaci* (Table 2) only a short distance downstream from the sampling site. Furthermore, *A. astac*i spores are alive and active and will more likely withstand chemical and biological processes in the water that lead to degradation of eDNA (Laurendz 2017), compared to cells shed from crayfish, a group reported to release only a very low amount of eDNA (Rice et al. 2018).

Strand et al. (2019) monthly monitored a watercourse for more than a year during an ongoing crayfish plague outbreak in Norway. There, the very scarce population of *P. leniusculus* that had caused the plague outbreak was detected by eDNA only in July and October, concurring with the presumed periods of moulting and reproduction, when more eDNA from the crayfish is likely to be released to the water. Dunn et al.

(2017) examined the relationship between eDNA concentration and crayfish biomass and were able to detect a relationship only when female *P. leniusculus* crayfish were ovigerous. Laurendz (2017) found no clear correlation between number of crayfish and eDNA emission in aquaria experiments with *P. leniusculus*, but observed peaks during moulting and huge quantitative variation depending on various environmental and biological factors. Similarly, Buxton et al. (2017) observed peaks of eDNA of the great crested newt (*Triturus cristatus*) towards the end of the adult breeding period and when newt larval abundance was at its highest. While studying seasonal variation of eDNA emission by freshwater pearl mussel (*Margaritifera margaritifera*), Wacker et al. (2019) measured the highest concentrations of eDNA in August, corresponding to the period these mussels release large amounts of larvae into the water. These studies and our results demonstrate that sample number, coverage, season, inhibition and other environmental factors can substantially influence the results and that eDNA methods may fail to detect elusive or rare targets. A robust knowledge of the biology of the target species is thus required for improving sampling success. In our study, although using large volumes that to some degree compensate for few samples, we would most likely increase the detection success with more samples.

A useful tool to help determine the number of samples required for maximising detection probability could be occupancy modelling. Schmidt et al. (2013) analysed data obtained while examining the presence of the chytrid fungus *Batrachochytrium dendrobatidis*. Based on an index similar to "catch-per-unit-effort", which is also obtainable for crayfish, they were able to calculate the amount of samples required for a detection probability to exceed 95%. Dougherty et al. (2016) used relative abundance and site characteristics as covariates to model the detection probability for *F. rusticus* using eDNA sampling. A similar tool for occupancy modelling, an R package for multiscale occupancy modelling of eDNA data, was recently presented by Dorazio and Erickson (2017).

Detection of the host-pathogen complex

In the screening of crayfish habitats, we successfully managed to detect eDNA of European noble crayfish and all three North American crayfish species investigated in this study. Here, we infrequently detected eDNA of the crayfish plague pathogen *A. astaci* together with the three investigated non-native crayfish species. More commonly, only eDNA from non-native crayfish was detected alone, suggesting low prevalence and infection load or possibly even absence of the pathogen (as also corroborated by analyses of the host crayfish tissues).

The eDNA monitoring methodology has been promoted as a reliable, non-invasive, ethical and animal welfare-friendly alternative to cage monitoring for early detection of crayfish plague (Wittwer et al. 2017; Strand et al. 2019). Indeed, when eDNA fails to detect *A. astaci*, although present at the location, it is likely that the pathogen spore concentration is too low to infect caged susceptible crayfish anyway. Strand et al. (2019) demonstrated that eDNA monitoring reveals the presence of *A. astaci* in the water earlier

than cages with live crayfish put out for disease surveillance. According to Strand et al. (2019), the simultaneous monitoring of native and non-native crayfish also provides additional information on habitat status, which otherwise requires trapping surveys.

We never detected eDNA from *A. astaci* together with native *A. astacus*, which is a good sign for the habitat status for these locations. However, in a few locations, eDNA from both native and non-native crayfish co-occurred. This could, in some cases, result from passive downstream transport of eDNA (Deiner and Altermatt 2014; Rice et al. 2018) from one of the target species that was geographically separated – even with migration barriers. However, in other cases it could reflect co-existence of native and non-native crayfish in the absence of infected crayfish carriers, or with very low *A. astaci* prevalence in the non-native crayfish population. In the latter case, it might only be a matter of time before the low-prevalent crayfish plague agent eradicates the native population. In a Norwegian lake, populations of *A. astacus* and *A. astaci*-carrying *P. leniusculus* presumably occurred at the same time for more than a decade before crayfish plague struck the native population (Vrålstad et al. 2011, 2014). This might be explained by low infection pressure and geographic separation within the lake.

The observed co-occurrence of eDNA from *A. astacus* and *F. limosus* in two locations, as well as *A. astacus* and *P. leniusculus* in one location, could suggest a possible syntopic presence of native and non-native species, although in at least one of the cases (location 10), downstream transport of *A. astacus* eDNA from a population upstream of the *F. limosus* population (location 9) is more likely. However, co-existence can occur in the absence of *A. astaci* infection in the non-native population. This has been thoroughly documented in Central Europe for *F. limosus* populations co-occurring with *A. astacus* (Schrimpf et al. 2013) and also for *P. leniusculus* populations co-occurring with *A. astacus* in Denmark (Skov et al. 2011). In our study, 70% and 80% of the *P. leniusculus* and *F. limosus* locations did not yield positive eDNA results for *A. astaci*, respectively. However, the number of individuals directly tested by us for infection was too low to conclude about the absence of the pathogen even at places where none was detected (see Schrimpf et al. 2013).

The co-occurrence of NICS in urban waters, represented by an inner-city lake (30 – Hundekehlensee) and a thermal stream (31 and 32 – Barát stream and its thermal inflow), demonstrates the importance these habitats play for the spread of NICS. The ornamental pet trade has been shown to be a major introduction pathway for non-native crayfish species into Europe (Peay 2009b; Chucholl 2013) and the species found at these locations are available through the pet trade (Mrugała et al. 2015). Additionally, eDNA of the crayfish plague pathogen *A. astaci* was detected at all three locations. Our findings highlight both the risks emanating from these habitats as well as the possibilities of monitoring similar habitats using eDNA.

Methods and sample strategies

The use of eDNA plays an important role in the present efforts to introduce advanced molecular tools into monitoring and bio-assessment of aquatic ecosystems (Leese et

al. 2016). This is particularly important with regard to the protection, preservation and restoration of aquatic ecosystems, which for European Union countries is legally binding through the Water Framework Directive (EU directive 2000/60/EC). Current approaches are still largely based on traditional sampling of organisms followed by identification by morphology, which is time-consuming and error-prone due to the varying and diminishing taxonomic expertise (Leese et al. 2016). While metabarcoding of environmental samples is the most promising approach for bioassessment and biodiversity inventory studies (de Vargas et al. 2015; Visco et al. 2015; Fujii et al. 2019), the more targeted qPCR approaches are specifically relevant for the monitoring of rare and red-listed native species and/or harmful invasive species of particular focus.

For both approaches, sampling strategies are of great importance for the quality and outcome regarding results. The choice of sample method, filter and volume might be of vital importance for maximising the detection probability of rare targets (Strand et al. 2014; Kumar et al. 2019). Crustaceans are more challenging to detect (Forsström and Vasemägi 2016; Rice et al. 2018) than fish, for example, that shed multiple sources of eDNA into the water (Jo et al. 2019). It appears, therefore, that efficient eDNA sampling for crayfish and their pathogen requires a substantially larger volume of water than for fish and amphibians. However, we are not aware of any study directly comparing these organisms.

The cost of the sampling equipment, as used for example in Strand et al. (2014, 2019) or Thomas et al. (2018), may be a limiting factor that prevents collection of suitable samples by a wider body of stakeholders. While conducting the fieldwork, we thus also evaluated the applicability of a robust, easy-to-use and low-cost version of the eDNA sampling equipment, based mostly on items readily available in garden stores and hobby markets. Most importantly, we exchanged the costly Masterflex E/S portable peristaltic pump-based sampler (retail price exceeding 2000 USD) with the drill-powered pumping system (ca. 26 USD without drill). This low-cost alternative provided very satisfactory results since it was possible to filter the same amount of water as sampled with the Masterflex E/S sampler and the target organisms were usually detected where expected. The difference between the two systems, which use exactly the same filter, is that water is pumped through the filter with suction, rather than pressure, since the filter is situated at the front of the drill-pump system. All parts of the entire setup can be detached and disinfected and the easy-to-replace filter cups eliminate issues with potential carry-over contamination. The low price of the equipment is a particularly important benefit for various stakeholders with limited budgets (e.g. nature conservancy agencies, NGOs, fishery managers).

Compared to the traditional methods used to determine presence or absence of crayfish which consist of either manual searching or trapping, this method requires less time in the field at each sampling site and it allows for sampling at locations unsuitable for traditional monitoring. For example, some of the sampling points visited by us were inaccessible for manual searching crayfish and would have required trapping or scuba diving, neither of which was possible during the fieldwork for this study. The eDNA methodology also enables the user to detect crayfish species when only small-sized in-

dividuals which might neither be caught in traps nor easily detected by manual search are dominant. Additionally, the extracted eDNA filter samples contain a broad variety of species from each location, both microorganisms and macroorganisms, and can be, at a later date, screened for entirely different targets (Dysthe et al. 2018). There is thus a potential for savings of both effort and costs if relevant stakeholders synchronise and/or collaborate on the eDNA sampling for multiple research and monitoring purposes.

Conclusions

The eDNA method based on targeted species-specific qPCR is suitable for detecting several invasive and native crayfish species as well as the crayfish plague pathogen in relevant habitat types in Central and Western Europe. The assays presented here performed well and yielded results that mostly corroborated our knowledge on the presence of native and non-native crayfish in the visited habitats.

It is particularly the positive data on the presence of crayfish and crayfish plague that yield valuable information, while negative results have to be interpreted with great caution. The latter should preferably be followed up with analyses of more samples collected in suitable periods, taking into account the time of year, temperature, water flow and the biology of the target species. This is of paramount importance if the absence of a specific species needs to be unambiguously established.

Including further assays of other crayfish species native to Central Europe, such as the stone crayfish, into this already broad panel will enable relevant stakeholders and authorities to use this method as a routine monitoring tool for all relevant crayfish species or in preparation of restocking operations.

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Supplementary material 1

Table S1

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: details on localities

Explanation note: Detailed information about the eDNA sampling sites visited during the study.

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Link: https://doi.org/10.3897/neobiota.58.49358.suppl1

Supplementary material 2

Table S2

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: species list

Explanation note: List of crayfish species used for in-vitro testing of the assay specificity.

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Link: https://doi.org/10.3897/neobiota.58.49358.suppl2

Supplementary material 3

Table S3

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: data for methods

Explanation note: Standard dilutions from crayfish genomic DNA.

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Link: https://doi.org/10.3897/neobiota.58.49358.suppl3

Supplementary material 4

Table S4

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: detailed results

Explanation note: Overview of the qPCR results, eDNA copy number estimation and PCR inhibition.

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